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Effect of phosphodiesterase type 4 inhibitor rolipram on cyclophosphamide-induced cystitis in rats

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Abstract

Cyclophosphamide induces a severe haemorrhagic cystitis characterized by bladder overactivity. The study was conducted to examine effects of a phosphodiesterase 4 (PDE4) inhibitor rolipram on bladder overactivity in rats with cyclophosphamide treatment. 42 female Wistar rats were used. 30 rats received a single i.p. injection of cyclophosphamide, and after 72 h, bladder function was evaluated by (1) in vitro preparations of whole bladders and (2) cystometry with continuous saline infusion under urethane anesthesia. Cyclophosphamide-treatment dramatically potentiated the basal spontaneous contractions of isolated whole bladders compared to control rats. Atropine, guanethidine or suramin was ineffective on the spontaneous contractions whereas nifedipine completely abolished. Rolipram (5–80 μM) induced a significant concentration-dependent decrease on the amplitude, frequency (contractions/min) and area under the curve of spontaneous contractions. Carbachol elicited phasic contractions superimposed on a tonic contraction. Rolipram caused a relaxation on the tonic contraction whereas it could not affect the phasic contractions induced by carbachol. In anesthetized rats, during continuous infusion cystometry, intercontraction interval was significantly shorter in cyclophosphamide-injected rats than in control rats. Rolipram at 5–40 μM has no significant effect on the intercontraction interval and contraction pressure while it significantly decreased pressure threshold. At 80 μM, it significantly decreased the intercontraction interval and contraction pressure. In conclusion, PDE4 inhibitor rolipram caused a significant decrease on the amplitude, frequency and area under the curve of basal spontaneous contractions in cyclophosphamide-treated rats, at doses that have no effect on the carbachol-induced phasic contractions and cystometric parameters. PDE4 inhibitors may be considered as an attractive strategy for the treatment of cyclophosphamide-induced bladder overactivity.

Keywords: Phosphodiesterase 4 inhibitors; Rolipram; Cyclophosphamide; Urinary bladder; Bladder overactivity; Cystitis

1. Introduction

Cyclophosphamide is a chemotherapeutic drug which is effective in the treatment of neoplastic diseases. However, this drug may induce a severe haemorrhagic cystitis. It is well established that this cystitis involves bladder overactivity characterized by decreased bladder capacity and increased micturition frequency (Stillwell and Benson, 1988; Ahluwalia et al., 1994). Cyclophosphamide-induced cystitis may require improved treatment strategies which relates to overactivity and inflammation (Ozawa et al., 1999; Yoshimura and Chancellor, 2002).

The role of Cyclic nucleotides (cAMP and cGMP) in modulation of detrusor contractile activity has been well shown (Truss et al., 1996a; Gillespie, 2004b). cAMP and cGMP are synthesized by adenylyl and guanylyl cyclases which are hydrolysed by the phosphodiesterase enyzmes (PDE). The intensity and the duration of the intracellular cyclic nucleotides are regulated by PDEs. There are 11 distinct PDE families isozymes. Among these PDE families, PDE1,-2,-3,-4,-5, and -9 have been expressed human bladder (Truss et al., 1996b; Rentore et al., 2003). It was suggested that detrusor relaxation is mainly mediated by the cAMP pathway rather than cGMP pathway. Some recent papers showed that PDE4 (high-affinity cAMP-specific PDE) inhibition suppressed rhythmic bladder

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contractions in human (Oger et al., 2007), guinea pig (Gillespie, 2004b) and rat (Synder et al., 2005). Nishiguchi et al. (2007) demonstrated that a PDE4 inhibitor could suppress the detrusor overactivity in rats with bladder outlet obstruction.

In view of these studies, it might be expected that PDE4 inhibition may be useful in the suppression of cyclophosphamide-induced bladder overactivity. However, to our knowledge, effects of PDE4 inhibitors on bladder overactivity in rats with cyclophosphamide-induced cystitis have not been reported. Thus, in the present study we aimed to examine the effect of rolipram, a PDE4 inhibitor on the spontaneous contractions in the isolated whole bladder of rats with cyclophosphamide-induced cystitis. Because, an increase of this spontaneous contractions may play a role in the pathophysiology of detrusor overactivity and these increased spontaneous contractions measured in vitro may correspond to the non-voiding contractions of the overactive bladders in vivo (Gillespie, 2004a; Szigeti et al., 2005). We also used cystometry with continuous saline infusion under urethane anesthesia to test the effect of rolipram on voiding function in normal and cyclophosphamide-injected rats.

2. Methods

2.1. Animals

42 adult female Wistar rats (200–250 g) were used throughout the experiments. The experimental procedures were approved by the animal care committee of the University of Çukurova (TIPDAM) and the studies were carried out in accordance with the Principles of laboratory animal care (National Institutes of Health guideline; publication No. 86-23, revised 1984). All animals were kept under standard laboratory conditions (12 h light / 12 h dark). 30 rats received a single i.p. injection of cyclophosphamide (150 mg/kg) dissolved in saline in a concentration of 40 mg/ml. In control rats ($n=12$), saline was injected i.p. After 72 h, the rats were prepared for cystometry ($n=18$) and in vitro whole bladder ($n=24$) experiments.

2.2. In vitro whole bladder preparation

Rats were anesthetized with halothane and sacrificed by cervical dislocation. We used the previous techniques for whole bladder preparation (Ng et al., 2006). The bladder was exposed by a midline abdominal incision and removed from the abdomen by cutting at the bladder neck. A 26-gauge needle was inserted at the bladder neck and tied with 5-0 silk sutures. The needle was connected to an infusion pump and pressure transducer via polyethylene tubing and a 3-way stopcock. The needle and tubing were filled with Krebs solution (113 mM NaCl, 19.8 mM $NaHCO_3$, 11.1 mM dextrose, 1.2 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $MgCl_2$, 1.7 mM $CaCl_2$). The bladder was placed between two platinum stimulating electrodes inside an organ bath filled with 37 °C Krebs solution and bubbled with 95% O_2/5% CO_2. Bladder pressure was recorded by a data acquisition software (BIOPAC MP30 Systems, Inc.). After a 30 min equilibration period, the bladder was filled slowly with Krebs solution in 50 μl increments during intermittent electrical field stimulation (50 V, 1.5 ms, 10 Hz for 15–30 s) to determine the bladder volume necessary to produce maximal bladder contractions. Field stimulation was delivered by a Grass S88 stimulator (Grass Instruments, Quincy, MA). The distended bladder was washed three times with 15 ml of fresh Krebs, equilibrated for another 30 min and then drug treatment was started. We used the 5 min intervals within the 10 min observation period to calculate the mean amplitude and frequency of the spontaneous contractions, after a drug was given. The peak amplitude of the spontaneous contractions was normalized as a percentage of the maximal K^+ evoked contraction amplitude. The K^+ evoked contraction was induced at the end of the experiments by bath solution containing 80 mM KCl. Frequency was determined by counting the number of contractions over a 5 min interval. In some experiments carbachol (1 μM) was applied to elicit the cholinergic contractions. In the experiments, the effects of various agents, including an adrenergic neuron blocker guanethidine (2 μM); a cholinergic receptor antagonist atropine (2 μM); a purinergic antagonist suramin (100 μM); a L-Type calcium channel blocker nifedipine (1 μM) or a PDE4 inhibitor rolipram (5–80 μM) were examined on the baseline spontaneous contractions or carbachol-induced contractions.

2.3. Cystometrogram recording

Animal were anesthetized with subcutaneous injection of urethane (1.2 g/kg). The urinary bladder was exposed via a midline abdominal incision and was catheterized. A PE-50 catheter, the bladder end of which was heated to create a collar, was inserted through a small incision in the bladder dome, and a suture was tightened around the collar. The other end was connected via a T-stopcock to a pump for continuous infusion of physiological saline and to a pressure transducer to record bladder pressure. Physiological saline was infused at room temperature into the bladder at a constant rate of 0.04 ml/min to elicit repeated voiding responses. In all experiments, control cystometrograms were recorded for about 2 h prior to intravesical drug administration. The parameters evaluated were amplitude (maximum bladder pressure during micturition), intercontraction interval (the time between two voiding cycles), pressure threshold (bladder pressure immediately prior to micturition) and basal pressure (the lowest bladder pressure during filling). Rolipram (5–80 μM) was administrated in saline into the bladder for 1 h. Cystometrogram parameters were monitored for 1 h and compared with the recordings before drug application.

2.4. Drugs

Carbachol, atropine, nifedipine and suramin were dissolved in distilled water. Rolipram was dissolved in ethyl alcohol (final concentration in the bath medium was 0.1%). At the concentrations used, ethyl alcohol had no effect on bladder activity. Drugs were obtained from Sigma Chemical Co., St. Louis, MI.

2.5. Statistical analysis

The spontaneous contractile activity was quantified by the calculation of the maximal amplitude (cm H_2O), the frequency (contractions per min) and the area under the curve (AUC). Amplitude and developed tension were expressed as a percentage of basal responses before drug application or KCl induced contraction at the end of each experiment. AUC was presented as cm H_2O min or percentage of basal responses. All data are expressed as mean±S.E.M. Data were analyzed by Student's *t* test. A *P* value of less than 0.05 is considered significant. Statistical analysis was performed with a GraphPad Prism software (San Diego, CA, USA).

3. Results

3.1. Changes on the spontaneous contractions of isolated whole bladder after cyclophosphamide treatment

We first compared the profile of spontaneous contractions between isolated whole bladders obtained from control and cyclophosphamide-injected rats. Most of control rats showed very small amplitude spontaneous contractions or no contractions. However, cyclophosphamide-treatment caused a dramatically enhancement on the amplitude of spontaneous contractions compared to control rats ($P<0.05$) (Figs. 1 and 2). The mean amplitude of the spontaneous contractions was 0.74±0.04 cm H_2O in control rats ($n=6$) and 3.7±0.2 cm H_2O in cyclophosphamide-treated rats ($n=6$) (Fig. 2). Also, the frequency and AUC of spontaneous contractions were significantly greater in cyclophosphamide-treated rats than in control rats (Fig. 2).

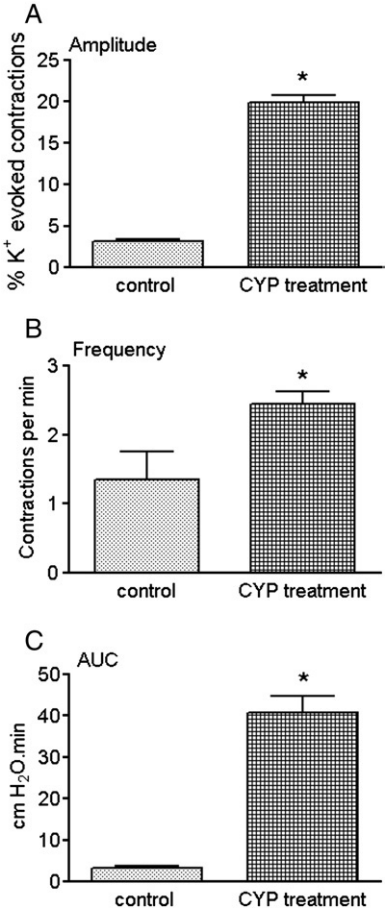

Fig. 2. Changes in amplitude, frequency and AUC of spontaneous contractions after cyclophosphamide injection. Amplitude is presented as percentage of K^+-evoked contractions; frequency, contractions per min; AUC, area under the curve. Results are expressed as mean±S.E. ($n=6$–8). *$P<0.05$, shows significant difference from control rats.

3.2. Effect of guanethidine, atropine, suramin or nifedipine on the spontaneous contractions of isolated whole bladder in cyclophosphamide-treated rats

2 μM guanethidine (an adrenergic neuron blocker), 2 μM atropine (muscarinic antagonist) and 100 μM suramin (purinerjic antagonist) did not affect the amplitude, frequency and AUC of the spontaneous contractions in cyclophosphamide-treated rats (Table 1). On the other hand, L-type calcium channels blocker nifedipine (1 μM) completely abolished the spontaneous contractions (Table 1).

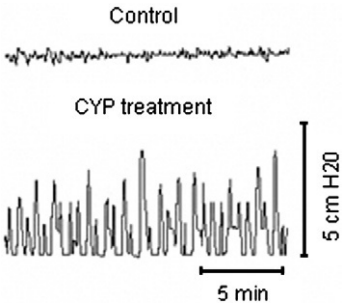

Fig. 1. Representative tracings showing the dramatic enhancement on the spontaneous contractions after cyclophosphamide injection.

3.3. Effect of rolipram on the spontaneous contractions of isolated whole bladder in cyclophosphamide-treated rats

Rolipram (5–80 μM) induced a significant concentration-dependent decrease on the amplitude, frequency and AUC of spontaneous contractions in cyclophosphamide-injected rats

Table 1
The ineffectiveness of atropine, guanethidine and suramin on spontaneous contractions of isolated whole bladder preparation in cyclophosphamide-treated rats

	Amplitude (%)	Frequency (%)	AUC (%)
Atropine (2 μM)	95.8±7.2	94.6±14.8	92.2±7.7
Guanethidine (2 μM)	97.9±6.3	98.5±11.6	99.5±8.6
Suramin (100 μM)	103.8±7.9	111.6±11.7	93.3±6.2
Nifedipine (1 μM)	0.0[a]	0.0[a]	0.0[a]

Nifedipine completely suppressed the spontaneous contractions.
AUC=area under the curve; frequency=contractions per min. The results are presented as percentage of the basal responses before the drug application (mean±SE, $n=6$). [a] $P<0.05$, shows significant difference from basal responses before drug application.

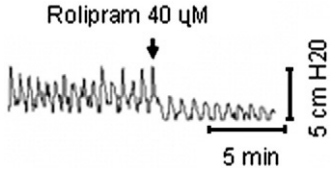

Fig. 3. Representative tracing showing the effect of 40 μM rolipram on the spontaneous contractions of isolated whole bladder preparation in cyclophosphamide-treated rats.

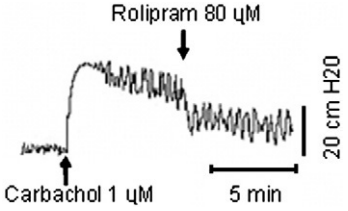

Fig. 5. Representative tracing showing the effect of 80 μM rolipram on the tonic and spontaneous contractions induced by 1 μM carbachol in cyclophosphamide-treated rats.

($P<0.05$; Figs. 3 and 4). The effect of rolipram on the spontaneous contractions was reproducible. Washing out rolipram with fresh Krebs solution could restore the level of spontaneous contraction back to the pre-rolipram state. The solvent of rolipram was ineffective on the spontaneous contractions in control and cyclophosphamide-treated rats (Fig. 4).

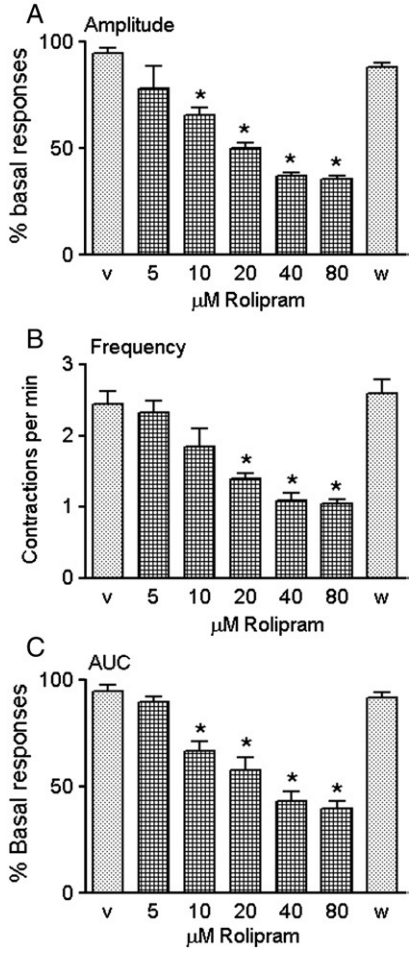

Fig. 4. Effect of rolipram (5–80 μM) on the basal spontaneous contractions of isolated whole bladder preparation in cyclophosphamide-treated rats. "v" column represents the results in the presence of ethyl alcohol (final concentration in the bath medium was 0.1%). 'w' represents washout. AUC, area under the curve. Amplitude and AUC are presented as percentage of basal responses. Results are expressed as mean±S.E.M. ($n=12$).*$P<0.05$, shows significant difference from basal responses.

3.4. Effect of rolipram on tonic and phasic contractions of isolated whole bladder induced by carbachol in cyclophosphamide-treated rats

1 μM, carbachol elicited tonic contractions followed by large amplitude and low frequency phasic contractions superimposed on an elevated basal tone in cyclophosphamide-treated rats (Fig. 5). The amplitude and AUC values of phasic contractions induced by carbachol were dramatically bigger than basal spontaneous contractions (amplitude increased to 251.2±43.7% of the basal spontaneous contractions; $P<0.05$). Rolipram (5–80 μM) caused a significant relaxation on carbachol-induced tonic contraction (% relaxation was 51.5±10.1 at 80 μM; $P<0.05$) (Fig. 5). On the other hand, rolipram at 5–80 μM did not affect the amplitudes, frequency and AUC of phasic contractions superimposed on elevated tone with carbachol (Fig. 5 and Table 2). These phasic contractions could be completely abolished by atropine (not shown). The solvent of rolipram was ineffective on carbachol-induced contractions (not shown).

3.5. Changes in cystometrogram parameters after cyclophosphamide treatment

During continuous infusion cystometry under urethane anesthesia, intercontraction interval was significantly smaller in cyclophosphamide-injected rats (4.98±0.71 min, $n=6$) than in control rats (9.89±0.74 min, $n=6$) (Fig. 6). The amplitude of voiding contractions increased compared to control rats but this increase was not statistically significant (data not shown).

3.6. Effect of rolipram on cystometrogram parameters in cyclophosphamide-treated rats

Rolipram at concentrations of 5–40 μM, did not elicit any significant changes on intercontraction interval and amplitudes

Table 2
The ineffectiveness of rolipram at the highest concentration (80 μM) on the spontaneous contractions induced by 1 μM carbachol in cyclophosphamide-treated rats

	Amplitude (%)	Frequency (%)	AUC (%)
Rolipram (80 μM)	105.7±14.6	101.1±7.91	107.6±9.42

AUC, area under the curve; frequency, contractions per min. The results are presented as percentage of the carbachol-induced spontaneous contractions before rolipram application (mean±SE, $n=12$).

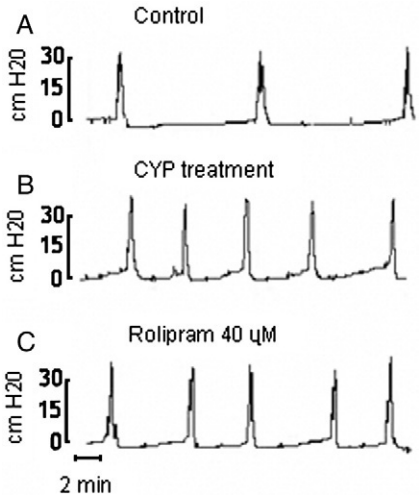

Fig. 6. Representative tracings showing the changes in voiding function during continuous infusion cystometry under urethane anesthesia after cyclophosphamide injection (A, control rats; B, cyclophosphamide-treated rats) and the ineffectiveness of 40 µM rolipram (C) on the voiding function in cyclophosphamide-treated rats.

of bladder contractions (Figs. 6 and 7; in Fig. 6, data was given for 40 µM rolipram). However, at highest concentration of 80 µM, rolipram significantly decreased the intercontraction interval and amplitudes of bladder contractions (Fig. 7). Rolipram at 20–80 µM caused a significant decrease on the pressure threshold in a concentration-dependent manner (Fig. 7). After washout of rolipram, pressure threshold increased to the previous level before rolipram application. At 10–80 µM, rolipram tended to decrease baseline pressure but these changes were not statistically significant (not shown). Also, in control rats, rolipram at low concentrations of 5–40 µM and its solvent do not have any significant effect on cystometrogram parameters while at 80 µM, rolipram significantly affect the cystometrogram parameters (not shown).

4. Discussion

The present experiments revealed that a PDE4 inhibitor rolipram caused a significant concentration-dependent decrease on the amplitude, frequency and AUC of spontaneous contractions in isolated whole bladder preparation of cyclophosphamide-treated rats without altering the amplitude of voiding contractions and the voiding interval during continuous cystometry in anesthetized rats. These findings suggest that the PDE4 inhibitor rolipram suppresses bladder overactivity in cyclophosphamide-induced cystitis.

In agreement with previous studies, cyclophosphamide treatment resulted in bladder overactivity (Ozawa et al., 1999; Jang et al., 2006). The intercontraction interval decreased and contraction pressure increased during continuous cystometry in anesthetized cyclophosphamide-treated rats. Also, cyclophosphamide-treatment dramatically potentiated the spontaneous contractions of isolated whole bladders compared to control rats. The amplitudes, frequency and AUC were significantly greater than in control rats. These increased spontaneous contractions measured in vitro may correspond to the non-voiding contractions of the bladder overactivity in vivo (Mills et al., 2000; Szigeti et al., 2005). It was well shown that the spontaneous contractions of in vitro whole bladder preparations are most likely myogenic in origin and they can be modulated by activation of various types of receptors (adrenergic, muscarinic and purinergic) (Levin et al., 1986; Sugaya and de Groat, 2002; Drake et al., 2003; Gillespie et al., 2003, 2005; Szell et al., 2003; Ng et al., 2006). Nevertheless, the exact mechanisms responsible for this spontaneous activity have not yet been determined. In the present study, guanethidine, atropine and suramin did not affect the variables of spontaneous contractions in cyclophosphamide-treated rats. These findings may suggest that the enhancement on the basal spontaneous contractions of isolated whole bladder in cyclophosphamide-treated rats is not due to an activation of adrenergic, muscarinic or purinergic mechanism. Our data may suggest that the basal spontaneous contractions of isolated whole bladder in

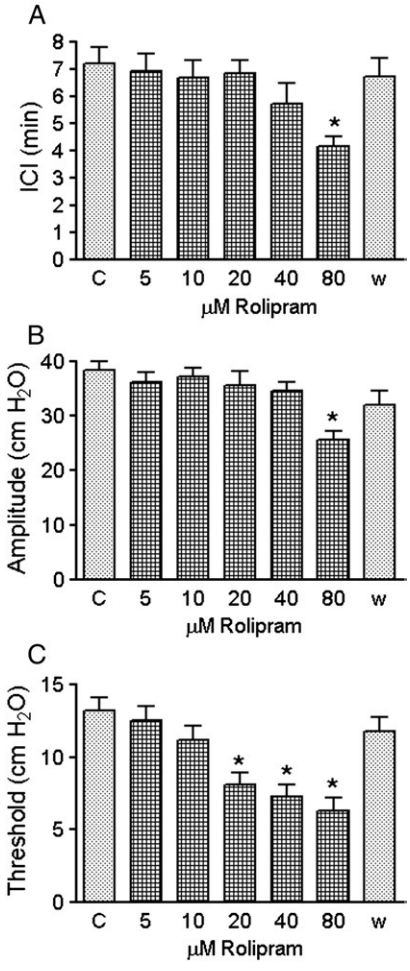

Fig. 7. Effect of rolipram (5–80 µM, intravesically) on the cystometrogram parameters during continuous infusion cystometry under urethane anesthesia in cyclophosphamide-treated rats. "C" column represents the results before intravesically rolipram application. 'w' represents washout. A, intercontraction intervals (ICI); B, contraction amplitude; C, pressure threshold. Results are expressed as mean±S.E.M ($n=6$). *$P<0.05$, shows significant difference from control responses.

cyclophosphamide-injected rats may be myogenic in origin (Brading, 1997). On the other hand, inhibition of L-type calcium channels by nifedipine completely inhibited spontaneous contractions. This result may indicate that the spontaneous contractions in cyclophosphamide-injected rats are dependent on calcium entry through voltage-dependent calcium channels (Buckner et al., 2002; Ng et al., 2006).

A PDE4 inhibitor rolipram caused a significant concentration-dependent decrease on the amplitude, frequency and AUC of spontaneous contractions in isolated whole bladder preparation of cyclophosphamide-treated rats. Such a suppression effect of a PDE4 inhibitor has previously been reported in human detrusor strips (Oger et al., 2007) and in whole guinea pig bladders (Gillespie 2004b). In these papers, it was demonstrated that PDE4 enzymes are functionally associated with the mechanism underlying the regulation of phasic myogenic activity of bladder tissue. Andersson (1999) suggested that during bladder filling, the stimulation of β-adrenoceptors by noradrenaline released from sympathetic nerve terminals causes an elevation of cAMP and relaxation of bladder smooth muscle. Thus, inhibition of PDE4 enzymes could suppress spontaneous contractions by potentiating this sympathetic input to the bladder smooth muscle. In a recent study, Nishiguchi et al. (2007) demonstrated that a new selective PDE4 inhibitor, IC486051 effectively suppressed non-voiding contractions during bladder filling in rats with bladder outlet obstruction. Our results are in agreement with these previous studies and we can suggest that PDE type 4 activity plays an important role in the mechanism of the enhanced basal spontaneous contractions in rats with cyclophosphamide-induced cystitis.

On the other hand, rolipram caused a significant relaxation on the tonic contraction induced by a non-specific muscaric receptor agonist carbachol. Our findings are in agreement with the previous studies which were conducted in humans (Oger et al., 2007) and in different animal species (Truss et al., 1996a; Qiu et al., 2001; Synder et al., 2005). All these papers strongly suggest that PDE4 enzymes are involved in the regulation of the cAMP pathway on the carbachol-induced contractile activity. However, an interesting result was that rolipram could not affect the phasic contractions superimposed on the carbachol-induced tone, at doses caused a remarkable decrease on the amplitude, AUC and frequency of basal spontaneous contractions. These results are not in agreement with some previous papers that reported PDE-4 inhibitors can suppress phasic activity induced by carbachol in the guinea pig (Gillespie, 2004b) and rat (Qiu et al., 2001). Our results showed a marked difference in the sensitivity of basal spontaneous contractions in cyclophosphamide-irritated preparations and carbachol-evoked phasic contractions. This raises the possibility that difference between basal spontaneous activity and phasic contractions evoked by cholinergic agent in the bladder might be differentially sensitive to rolipram. Cyclophosphamide-induced activity could be mediated by spontaneous release of afferent neuropeptides from C-fiber nerves (Ozawa et al., 1999). Also, Oger et al. (2007) showed that the relaxing effect of rolipram on the carbachol-induced contractions was enhanced when cAMP levels were increased by forskolin. On the other hand, it is suggested that PDE4 isoenzymes are only weakly involved in the regulation of the voiding contractions which are mainly elicited by muscarinic receptor stimulation (Truss et al., 1996a, 1996b; Synder et al., 2005). In view of these reports, it can be suggested that the ineffectiveness of rolipram may be partly due to a possible weaker role of PDE4 isoenzymes in the regulation of phasic contractions evoked by carbachol than of basal spontaneous contractions. However, we need some future investigations about the mechanisms that generate different types of spontaneous activity and at what site PDE-4 functions such as interstitial cells (Lagou et al., 2006). We also need to demonstrate the roles of specific PDE isoenzyme in phasic and tonic contractions in normal and other overactive bladder models induced by cyclophosphamide in rats.

During cystometry, intercontraction interval significantly decreased in cyclophosphamide-treated rats compared to control rats. However, it was not observed a clear non-voiding activity. This may be due to anaesthesia since Streng et al. (2006) showed that anaesthesia affects the bladder activity. Rolipram at low concentrations did not affect the voiding parameters except pressure threshold in control and cyclophosphamide-injected rats. Our findings are in agreement with a recent paper published by Nishiguchi et al. (2007). In this paper, it was suggested that inhibition of PDE4 in the rats with bladder outlet obstruction has a minimal effect on voiding bladder contractions since sympathetic bladder efferents are inhibited during voiding. According to our results, it seems that bladder outlet obstruction induced activity and cyclophosphamide-induced have a similar sensitivity to PDE4 inhibitors. The finding that the amplitude of bladder voiding contractions in control and cyclophosphamide-treated rats was not changed by rolipram may indicate that the rolipram-induced suppression of bladder overactivity in isolated whole bladder may not be due to a direct effect on bladder smooth muscle. It may be possible that rolipram can affect the networks of interstitial cells. However, rolipram at high concentrations of 80 μM increased the intercontraction interval and decreased the amplitude of voiding contraction in cyclophosphamide-treated rats. This may be due to the activation of other signaling pathways by rolipram (Uckert et al., 2002; Lagou et al., 2006; Nishiguchi et al., 2007).

In conclusion, rolipram caused a remarkable decrease on the amplitude, AUC and frequency of the spontaneous contractions in cyclophosphamide-treated rats, at doses that have no effect on voiding bladder contractions. Also, the present study showed a marked difference in the sensitivity of basal spontaneous contractions and carbachol-evoked phasic contractions in cyclophosphamide-treated rats. This raises the possibility that different types of spontaneous activity might be differentially sensitive to rolipram. Our results may give some insight to the mechanisms that generate different types of spontaneous activity and at what site PDE-4 functions.

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